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PROVISIONAL APPLICATION

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Transmitted herewith for filing is a PROVISIONAL APPLICATION of:

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For: MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION

Enclosed are:

X 47 sheets of specification
X 15 sheets of drawings (INFORMAL)
X A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27

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MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to detection and methods of treating cancer by utilizing the gene human type protein phosphatase 2C (PP2C) and gene products thereof, and preparing native and transgenic organisms in which the gene products encoded by the PP2C gene or its homolog in other species are artificially produced, or the expression of the native PP2C gene is modified and kits for the practice of the invention.

BACKGROUND ART

Gene amplification is one of the distinct abnormalities associated with malignant cells and transformed cell lines. This phenomenon is part of the genetic instability characterizing neoplastic cells and occurs rarely in normal cells. If the transformed phenotype can be reversed, i.e. the cells loose their neoplastic phenotype, thereby reinstating normal cellular growth the cancerous cells can be controlled providing a treatment.

Adeno associated viruses are members of the family of parvoviruses for which tumor suppressive properties have already been described in 1960 (for review see Rommelaere and Tattersal, 1990). They are a group of small viruses, with a ssDNA genome of approximately 5000 nucleotides, characterized by identical palindromic termini of 154 bases. The left part of the AAV genome encodes four multifunctional, overlapping,

non-structural proteins (Rep78, Rep68, Rep52 and Rep40) which are translated from differentially spliced mRNA driven by the P5 and P19 promoters. In the right part of the genome three overlapping capsid polypeptides (VP1 -VP3) are encoded from the P40 promoter (Berns, 1990; Leonard and Berns, 1994). These extremely small DNA viruses are represented in vertebrates by two genera, the autonomously replicating and the helper dependent parvovirus (Siegl et al., 1985).

The helper-dependent adeno-associated viruses (AAV) depend for their replication on coinfecting helper virus (Young and Mayor, 1979ab), or on conditions of genotoxic stress (Yakobson et al., 1987) and comprise agents infecting humans without apparent disease (Cukor et al., 1984). Helper viruses are adenoviruses (Atchison et al., 1965), herpes group viruses (Salo and Mayor, 1979) and vaccinia virus (Schlehofer et al., 1986). The helper viruses share the ability to induce chromosomal damage early in their infection cycle (Schlehofer and zur Hausen, 1982).

Anti tumor activity of adeno associated virus:

Tumor suppressive properties have been found for AAV (for review see Schlehofer, 1994). It has been shown that the development of tumors induced in rodents by adenoviruses, herpes viruses or by transplantation of cells transformed by these viruses could be inhibited by infecting the animal cells with AAV (Kirschtein et al. 1968; Mayor et al., 1973; de la Maza and Carter, 1981; Ostrove et al., 1981). The in-vivo findings of tumor suppression are paralleled by results showing inhibition of cellular transformation in-vitro. This could be shown for cells of different origin (hamster and mouse) transformed by viruses or by activated oncogenes. Compared with controls, cells infected with AAV or transfected with specific AAV DNA

sequences displayed decreased focus formation and saturation density indicating inhibition of transformation-associated traits (Casto and Goodheart, 1972; Katz and Carter, 1986; Hermonat, 1989; Hermonat, 1994).

In addition, there are seroepidemiologic findings in the human population, showing that cancer patients exhibit antibodies to AAV less frequently than matched control individuals. Three independent studies carried out in the USA (Mayor et al., 1976), Belgium (Sprecher-Goldberger et al., 1971) and Germany (Georg-Fries et al., 1984) using different serologic techniques, have found a high prevalence of antibodies to AAV (types 2, 3, and 5) in the normal population contrasting with a relatively low frequency of seropositivity in patients with cancer.

It would be useful to develop therapeutic methods for controlling cell transformation. As the above information indicates it is possible to reverse cell transformation or to specifically kill the transformed cell. It would be useful to find human cellular mechanisms that can be controlled to reverse cell transformation.

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, a method and kit of detecting cancer in a patient by detecting activity of the gene coding for human type protein phosphatase 2C (PP2C) and genetic polymorphisms thereof in a specimen containing cells isolated from the patient is disclosed. The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include

regulatory elements to control the expressibility of PP2C. The vector is then administered to the patient. Alternatively an antisense vector can be prepared.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-E are photomicrographs of Control (C) and MNNG (10 μg/ml) treated (T) CO60 and 9-3 cells which were trypsinized 72 hours after treatment and immobilized onto a glass-microscopic slide followed by the in-situ hybridization procedure. SV40 biotinylated probe was conjugated with fluorescein labeled Avidin (yellow). The chromosomes were stained with Propidium Iodide (red). The fluorescent signal was detected under a fluorescent microscope at 495nm.

FIGURE 2A-B are graphs of a FACS analysis of CO60 and two AAV/neo cell lines 913 and 916 as prepared for cell cycle analysis. 24 hours after seeding the cell were trypsinized and washed with PBS. The cells were resuspended in 1ml buffer containing 0.1% sodium citrate, 0.1% triton X-100 and 50 μg propidium iodide, and then processed in the FACS.

FIGURE 3A-D are photomicrographs showing in-vivo detection of programmed cell death. Treated cells or control cells were grown on pretreated coverslips in Petri dishes. 48 hours after seeding the coverslips were washed with PBS, pH 7.5 at 37°C for 5 minutes and covered with dye mix (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS pH 7.5), then placed upside down on a glass slide and immediately examined

by fluorescence microscopy. Note the shrinkage of the cytoplasm in treated 9-3.

FIGURE 4A-D are photomicrographs showing In-situ detection of programmed cell death in cells grown on pretreated coverslips in 60mm Petri dishes. 48 hours after MNNG treatment (2.0 μg/ml or 10 μg/ml MNNG for 9-3 and CO60 respectively) the cells were processed as described by Gavrieli et al. (1992) and Wijsman et al. (1993).

FIGURE 5 is a photograph of a Southern Blot analysis showing CHINT is associated with AAV integration in different AAV/neo cell lines. Southern blot analysis of different AAV/neo clones, CO60 DNA, digested with BglII, and hybridized with "CHINT" probe. 9-1, 2, 3, 4 and 5 on AAV/neo cell lines. 93R is a revertant that lost the whole chromosome containing the AAV. A6 is a mouse cell line.

FIGURE 6 is a photomicrograph showing AAV integration in 9-3 cells. 9-3 metaphase cells were immobilized onto glass microscope slides, followed by in-situ hybridization procedure. pJDT277 biotinylated probe was conjugated with Fluorescein labeled avidin (yellow). The chromosomes were prestained with Propidium Iodide. Arrows point to the AAV integration sites.

FIGURE 7 is a schematic representation of the organization of the integrated AAV and the flanking cellular sequences in 9-3 cells. A genomic library was prepared from C9-3 cells using the EMBL-4 lambda phage and scored for AAV positive clones. A clone of 13Kb-λSL9-1 was isolated and later subcloned to a blue-script vector. Plasmids pSL9-11 (13Kb), pSL9-8 (10Kb) and pSL9-6 (3Kb) were obtained as indicated in the figure.

FIGURE 8A-B shows (A) the homology between CHINT and a sequence from human DNA from cosmid DNA MMDB

containing the 5' end of the PP2C RNA and (B) the homology between MMDB and the human PP2C cDNA.

FIGURE 9A-B wherein (A) is a photograph of a Southern Blot Analysis showing AAV is adjacent to the gene coding to PP2C in 9-3 cells, The Southern blot analysis of genomic DNA, from CO60 and 9-3 cells digested by EcoRI, or XbaI was hybridized sequentially with the following probes: 1) AAV; 2) CHINT; and 3) Rat PP2C probes. The CHINT and the PP2C sequences are adjacent (4Kb EcoRI fragment). The AAV CHINT and PP2C are in a close proximity in 9-3 cells (the 5.6Kb XbaI fragment). (B) pSL9-6 is adjacent to PP2C in the wild type Chinese hamster cells. BamHI digested RNA from the Chinese hamster neo cells was hybridized to pSL9-6 and PP2C probes. A common fragment of ~8.5Kb appeared in all cell lines including CO60. The same fragment hybridized also to CHINT probe (data not shown).

FIGURE 10A-C are photographs of a Southern blot analysis of DA3 (lane 8) and DA3J1-DA3J1 cells lines (lanes 1-7). Genomic DNA was digested with BglII. The blots were hybridized sequentially with an AAV/neo JDT277, pSL9-6 and PP2C PCR probes. A 4Kb fragment hybridized to the AAV probe and pSL9-6 probe in J3 (lane 3), J4 (lane 4) and J6 (lane 6). A fragment smaller than 4Kb hybridized to both AAV and PP2C probe in J1 (lane 1), J2 (lane 2), J5 (lane 5) and J6 (lane 6).

FIGURE 11 is a photograph which shows the alteration in PP2C mRNA in response to carcinogen treatment. Forty μg of total RNA were isolated from CO60 and C9-3 cells 12 hours after treatment with MNNG (7.5 μg/ml and 2.5 μg/ml respectively), and from untreated cells and fractionated on a denaturing gel (1.2% agarose/6.6% formaldehyde gel). The gel was blotted and hybridized consecutively with 32P-labeled rat PP2C cDNA (A) pSL9-1^{DNA} (B) and rRNA cDNA (C).

FIGURE 12A-D are photographs which show rescue of the transformed phenotype by transfection with PP2C cDNA. A full length cDNA encoding rat type 2C protein phosphatase (~2.4Kb) was isolated from rat embryo cDNA library in lambda Zap (kindly provided by M. Oren from the Weizmann Institute of Science). The cDNA is expressed from a CMV promoter. PP2C clone (5 μg) was stably transfected into AAV/neo cells (C9-3) (C9-3 passage 17) together with a gpt plasmid (5 μg) conferring resistance to mycophenolic acid (MPA). The plasmids were introduced using the calcium phosphate co-precipitation method. Following a three day expression period, $2x10^5$ cells were plated on soft agar with the selective agent MPA. A control plasmid containing the 3'UTR of non relevant gene (cyclin G) was cotransfected with the gpt plasmid. One month later the colonies were counted. In the figure A and B, control plasmid; C and D, PP2C DNA. PP2C transfected cells regained their capability to grow in soft agar.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention discloses a method of detecting cancer in a patient by detecting activity of the gene coding for human type protein phosphatase 2C (PP2C) and genetic polymorphisms thereof in a specimen containing cells isolated from the patient.

Polymorphisms are variants in the gene sequence. They can be sequence shifts found between different ethnic and geographic locations which, while having a different sequence, produce functionally equivalent gene products, isoforms. Polymorisms also encompass variations which can be classified as alleles and/or mutations which can produce gene products which may have an altered function. Polymorisms also encompass

variations which can be classified as alleles and/or mutations which either produce no gene product or an inactive gene product.

Samples can be biopsied material from suspected precancerous lesions or any tissue or bodily fluid which can be assayed for PP2C activity.

In an embodiment the detection of PP2C activity is by assaying the specimen for mRNA complementary to PP2C DNA including polymorphisms thereof with an assay selected from the group consisting of in situ hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

The present invention also provides for a kit for detecting PP2C activity. The kit includes molecular probes for mRNA for PP2C polymorphisms thereof and detection means for detecting the molecular probe and thereby the mRNA.

In an alternative method, the detecting of PP2C activity is by assaying the specimen for a PP2C gene product including polymorphisms thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays for phosphatase activity and protein truncation test.

The present invention also provides for a kit for detecting a gene product interacting with PP2C gene products. The kit can include an antibody which recognizes the gene products including polymorphisms thereof, and detection means for detecting the binding of the antibody thereby indicating the presence of the gene product.

The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will

specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2C. The vector is then administered to the patient. Alternatively an antisense vector can be prepared. In a preferred embodiment the AAV virus and/or CHINT sequences are used in the vector. CHINT is a cellular sequence which was recombined into the AAV in 9-3 cells; the sequence is set forth in Figure 8A. The vector can either reestablish regulatory control of PP2C expression thereby leading to differentiation or alternatively, differentiation and cell death.

There are several elements in AAV and CHINT that might be used to control PP2C expression:

1. PP2C has a very long 5' and 3' UTR (they are larger than the coding capacity). Specific folding of the RNA and interaction with specific sets of proteins might effect its expression dramatically. At certain stages there might different modes of folding and different proteins may interact with the RNA and thus regulate its expression.

2. The CHINT sequences involved in the integration have very interesting motifs which might be used for the site specific integration. Morever, applicant has preliminary data sugggesting that the CHINT fragment, possibly together with the AAV sequences adjacent to it, are responsible for the suppression of DNA amplification.

The invention further provides a method of preventing cancer by using an AAV vector for cancer prevention that only functions specifically in cells in which PP2C is improperly activated. The modified AAV will be used at high dose to infect healthy populations. The dormant integrated virus will interrupt PP2C expression and will either prevent the appearance or the initiation of tumor cells.

The present invention provides an antibody, either polyclonal or monoclonal, which specifically binds to a polypeptide/protein encoded by the PP2C gene. In preparing the antibody, either the entire protein or peptide sequences can be used as an immunogen as well as polymorphisms thereof. The invention further provides antibodies that will recognize the special structures at the 5'UTR or the RNA-proteins complexes responsible for the controlled expression of PP2C. Antibody which recognizes specifically the special RNA structures is also provided.

The antibodies may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the protein or peptide, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies, the technique involves hyperimmunization of an appropriate donor, generally a mouse, with the protein or peptide fragment and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and

the monoclonal antibodies harvested from the culture media for use.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow and Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, ß-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

The present invention provides vectors comprising an expression control sequence operatively linked to the nucleic acid sequence of the PP2C gene and portions thereof as well as polymorphic sequences thereof. The present invention further provides host cells, selected from suitable eucaryotic and procaryotic cells, which are transformed with these vectors.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), in Ausubel et al., *Current Protocols in*

Molecular Biology, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995) and Gilboa, et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

More specifically, such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage

vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

Recombinant methods known in the art can also be used to achieve the sense, antisense or triplex inhibition of a target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity.

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and

can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect

neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recomnbinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However,

local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration of of a PP2C vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the tumor with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as

liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

In a preferred embodiment, a virus vector based on modified AAV is used. AAV virus has been shown to integrate into the human genome in chromosome 19q13.3 but not into the PP2C gene. The human PP2C regulatory region has some of the motifs required for AAV integration, a slight manipulation of the viral AAV sequences will facilitate its integration to the PP2C in human.

The present invention provides for transgenic PP2C gene and polymorphic PP2C gene animal and cellular (cell lines) models as well as for knockout PP2C models. These models are constructed using standard methods known in the art and as set forth in United States Patents 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384,5,175,383, 4,736,866 as well as Burke and Olson, (1991), Capecchi, (1989), Davies et al., (1992), Dickinson et al., (1993), Huxley et al., (1991), Jakobovits et al., (1993), Lamb et al., (1993), Rothstein, (1991), Schedl et al., (1993), Strauss et al., (1993). Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide information.

To investigate the role of AAV in tumor suppression an AAV/neo virus JDT277 was introduced into SV40 transformed Chinese hamster (CO60 and OD4 cells) and mouse mammary tumor cells (DA3). CO60 is a cell line of SV40 transformed Chinese hamster embryo cell lines (Lavi, 1981). The OD cell line was established by transfection of Chinese hamster embryonic cells with origin deleted SV40 DNA (Lavi, 1985). The mouse DA3 cell line was derived from mammary tumors syngeneic to BALB/c mice (Sotomayor et al, 1991). JDT277 virus contains the portion of the AAV2 genome, which encodes the viral Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882, resulting in carboxy terminus truncated Rep proteins. The truncation of the rep proteins does not affect the ability of the AAV/neo virus to replicate in Adenovirus coinfected human cells.

Single colonies were isolated and amplified by serial passages in the presence of G418. The resistant cells were designated 9-1 to 9-5 for cells derived from CO60 cells and A20-A29 for cells derived from OD4 cells. The cell lines derived from the mouse cells DA3 were designated J1-J15.

Alterations of the transformed phenotype: Following AAV integration the cells lost several of their transformed characteristics.

1. Suppression of SV40 DNA amplification.

A characteristic trait of tumor cells is their capability to amplify DNA. CO60 cells are used as a model system to study gene amplification and SV40 amplification can be induced in the cells as a results of treatment with carcinogens (Lavi, 1981; Aladjem and Lavi, 1992). Following AAV/neo virus integration, the cells were incapable to amplify SV40. Most AAV/neo cell lines derived from CO60 cells lost their capability to amplify SV40 upon treatment with carcinogen in contrast to the parental CO60 cells (Tal Burstyn, 1993). An example for this suppression is presented in Figure 1. Extracts from AAV/neo cells derived from both OD4 and CO60 cells lost their capability to amplify SV40 in-vitro (Winocour et al., 1992; Tal Burstyn, 1993).

2. The cells harboring the integrated SV40 became highly sensitive to treatment with UV or MNNG. (Winocour et al., 1992).

3. The transformed cells lost their capability to grow in soft agar, a characteristic typical to transformed cells.

4. The cells displayed apoptotic phenotype as measured by

a) the cell cycle pattern (Figure 2),

b) the condensation and fragmentation of the chromatin (Figure 3) and cytoplasm condensation and fragmentation of chromation,

c) by the breaks in the chromosomal DNA (Figure 4).

The integrated AAV in the AAV/neo cells

Analysis of cell extract derived from the Chinese hamster A20-A29, 9-1 to 9-5 and the mouse DA3 derivatives for the expression of rep proteins by immunoblotting with anti-Rep antibodies demonstrated that in all cell lines the authentic Rep products were not present. In some cell lines a short protein, probably a truncated protein, reacted with the anti-Rep antibodies (Winocour et. al., 1992).

PCR analysis of most cell lines for the presence of the intact promoter region demonstrated that in most cell lines the promoter region of the Rep protein was reorganized as a result of deletions, insertions and

rearrangements of the AAV sequences thus eliminating the expression of the authentic Rep proteins.

Applicant focused on the analysis of one of the cell lines, 9-3, derived from C060 cells. As shown in Figure 6 the integrated AAV undergoes duplication in this cell line and the chromosome harboring the AAV contains two regions containing the integrated AAV genomes. Note that this duplication of AAV probably resulted from the massive rearrangement which occurred in the Chinese hamster genome following AAV integration. In all cell lines studied so far by in-situ hybridization (6 independent cell lines) the chromosome harboring the integrated AAV was altered and was different in many respects from all the typical Chinese hamster chromosomes, thus the identity of the chromosome could not be established.

The integrated AAV and flanking cellular sequences were cloned into a phage (Figure 7). As shown in Figure 7 the viral genome underwent several changes. Sequences downstream to the AAV p5 promoter were deleted and replaced by a cellular fragment "CHINT". In addition, deletions and rearrangements in the 5' portion of the AAV/neo genome were observed. In contrast, the region coding for the Neo gene and the 3' end of the viral genome remained intact. (Similar alterations were observed in all AAV/neo Chinese hamster and mouse cell lines tested).

The "CHINT" sequences were used as a probe to analyze AAV integration site in different AAV/neo Chinese hamster clones (Figure 5). In several AAV/neo clones there was a shift in this fragment suggesting that the size of this fragment was altered upon AAV integration. Some of the shifted bands also hybridized to AAV probe demonstrating that AAV indeed integrated into this region. A probe from the subclonned plasmid pSL9-6 derived from the flanking cellular sequences (Figure 7) was hybridized to the mouse DA3 AAV/neo cells and in most cases it was associated with AAV integration. These results suggest that the AAV integration site in Chinese hamster might be similar to the one in mouse cells (data not shown).

Sequence comparison using Genetic Computer Group Inc. software demonstrated that the CHINT sequences are 58.3% homologous to a sequence in the human chromosome 19q13.3. This human sequence is a part of a 106Kb fragment which was automatically sequenced and analyzed (Martin-Gallardo et al., 1992) (GENBANK accession number: M89651). The region which showed homology to the CHINT sequences was a part of the gene coding for human type protein phosphatase 2C (PP2C). According to the cDNA sequence of this gene the exact region homologous to CHINT is located upstream to the 5'UTR of PP2C (Figure 8) (GENBANK accession numbers: human PP2C S87759, rabbit PP2C S87757).

Using a PCR fragment derived by using two primers for PP2C, cDNA DNA from 9-3 was probed and a XbaI fragment was found which hybridized to AAV, CHINT and PP2C, and an EcoRI fragment was found which hybridized to PP2C and CHINT in CO60 DNA. Thus, PP2C is indeed localized in very close proximity to CHINT in CO60 cells and is at the integration site in 9-3 cells. In-situ hybridization confirmed this conclusion (Figure 9A).

In both Chinese hamster (CO60 and OD4) and mouse cells (DA3) a portion of the PP2C sequences was adjacent to the CHINT hamster sequence present in pSL9-6 (Figure 7) which was derived from the lambda clone of the AAV integration site. Thus in both the normal Chinese hamster and mouse chromosome PP2C is indeed localized at very close proximity, less than 4Kb from the sequences surrounding the integrated AAV (Figure 9B).

PP2C is a protein serine/threonine phosphatase (Cohen 1989). It is unique amongst phosphatases since it requires magnesium and is sensitive to certain phosphatase inhibitors such as okadaic acid (Cohen 1991). The PP2C family consists of two cytoplasmic isoenzymes in mammalian tissues (McGowan and Cohen, 1987) and at least three PP2C-like enzymes in yeast show the same enzymatic and biochemical properties.

The two mammalian isoenzymes are monomers but differ slightly in molecular mass (44KDa and 42KDa).

Little is known about the role of PP2C in the cell or about its native substrates mainly because of the lack of specific inhibitors for PP2C. In *Schizosaccharomyces pombe* it has been shown that PP2C-like enzymes are important for heat shock response and in osmoregulation (Shiozaki, 1995; Shiozaki, 1994). In *Saccharomyces cerevisiae* PP2C-like activity has been implicated in the regulation of tRNA splicing and cell separation (Robinson et al., 1994). In neural cells PP2C might have a role in the regulation of the Ca++-independent activity of Ca++/calmodulin dependent protein kinase II (Fukugana, 1993). Otherwise, information about PP2C is scarce.

PP2C might be a cell marker itself. We do not know anything yet about its expression in tumor cells. There is a manuscript suggesting that PP2C might have a role during myogenic differentiation. (Ohishi, 1992).

Furthermore, in mouse DA3 cells harboring the integrated AAV genome the AAV sequences were associated with PP2C or with fragment 6 or with both (Figure 10).

Unexpectedly, analysis of the PP2C mRNA in the AAV/neo cells demonstrated that the transcription of the gene was reduced upon treatment with DNA damaging agents in contrast to the parental SV40 transformed

cells in which PP2C was induced following the treatment (Figure 11).

The following is the densitometry analysis of the hybridization signals

	CO60 C	CO60 T	CO60 T / CO60 C	C9-3C	C9-3T	C9-3T / C9-3C
PP2C / rRNA	0.15	0.31	2.06	0.26	0.15	0.57

The transcription of PP2C in C9-3 was reduced upon treatment with MNNG in contrast to the parental SV40 transformed cells in which PP2C was induced following treatment.

The intact cDNA coding for PP2C was cloned from a cDNA library (provided by M. Oren, the Weizmann Institute of Science, Rehovot) and PP2C clone was stably introduced into the AAV/neo cells which lost the transformed phenotype following AAV-integration. The transformed features were rescued following the expression of the PP2C clone in the PP2C neo cells, the cells regained the properties of transformed cells, grew in soft agar and lost their apoptotic phenotype (Figure 12). Similar cells which were transfected in the same efficiency with a control plasmid containing a truncated cDNA of non relevant gene did not regain the capability to grow in soft agar.

From these studies it is apparent that PP2C has a key role in the initiation and/or maintenance of transformed cells.

PP2C might be a marker by itself. We do not know anything yet about its expression in tumor cells. There is a manuscript suggesting that PP2C might have a role during myogenic differentiation. (Ohishi, 1992).

PP2C appears to be important in development. The high conservation of the gene and protein throughout

evolution, the specific control signals including IRES (internal ribosome entry site) at the 5'UTR support this. The findings by other laboratories that AAV infection effects specifically tumor cells might have two explanations: 1) the virus does not infect normal cells or cannot integrate into their genome in a specific manner. 2) alternatively, if AAV integrates into PP2C in normal cells the disruption of this gene might not effect them. The fact that the inactivation of only one allele of PP2C is responsible for the changes in the transformed phenotype and that the introduction of a functional PP2C cDNA clone rescues the transformed phenotype suggest that PP2C is important indeed. Applicant also noticed that in highly tumorigenic Chinese hamster cells the whole chromosome carrying PP2C is duplicated 3,4, and even 5 times.

In human, on the same chromosome, in a close vicinity to PP2C there is an important tumor specific marker called cancer embryonic antigen (CEA), which appears in most tumor cells. Both genes are mapped to chromosome 19q13.3. Targeting the treatment to cells carrying a marker like CEA might help. (It might be possible that CEA by itself is not relevant to cancer but it is associated with the enhanced expression of PP2C or duplication of this chromosomal region contains both genes).

The above discussion provides a factual basis for the use of PPC2 in cancer detection and therapy. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompaning figures.

EXAMPLES

GENERAL METHODS:

General methods in molecular biology:

Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1994).

Generation of antibodies against the PP2C

According to the PP2C amino acid sequence a synthetic oligopeptide will be synthesized. This oligopeptide will be injected to rabbits in order to generate polyclonal antibodies against PP2C. Western blot's analysis will be performed using these antibodies.

PP2C activity assay

PP2C gene product is purified from the mouse cells by general procedure, and its activity will be assayed by its ability to dephosphorylate [32P] casein (McGowan and Cohen, 1988).

Antisense RNA

Artificial antisense RNA complementary to the PP2C gene is synthesized, and transfected to the mouse cells by the method of Inouye (1988).

Identification of unique changes in gene expression by differential display

In order to detect changes in the expression of cellular genes mediated by AAV integration, the differential display method is used (Liang and Pardee, 1992; McClelliand et al., 1995). This method is

directed toward the identification of differentially expressed genes among approximately 15,000 individual mRNA species in a pair of mammalian cell population such as infected and uninfected cells, and recovering their cDNA and genomic clones. The strategy of the method consists of the following steps: (1) Reverse transcription in fractions using a set of anchored primers, (2) amplification of cDNA species from each fraction using a set of arbitrary primers and anchored primers by labeled PCR, (3) electrophoretic separation of the resulting fragments on sequencing gel, (4) reamplification of fragments that are different in the two situations, cloning and sequencing, and (5) confirmation of differential expression by an independent RNA analysis technique.

Chromatin structure in latently infected cells

Higher order chromatin structure may affect the transcription of cellular genes. Judging by their increased sensitivity to digestion with Dnase I or micrococcal nuclease, transcriptionally active chromatin regions are less tightly packed than chromatin containing transcriptionally inactive genes. Chromatin will be partially purified and digested by micrococcal nuclease (Roth et al., 1990). Purified DNA fragments will be digested with a unique restriction enzyme to generate a series of fragments with one end defined by micrococcal nuclease and the other defined by the restriction enzyme. Fragments will be separated by agarose gel electrophoresis, transfred to nitrocellulose filters and probed with labeled DNA fragments. Naked DNA will be purified and processed similarly. Nucleosome position and nuclease sensitive regions will be inferred by comparison of fragments from naked DNA and chromatin.

The methylation state of genes can indicate chromatin changes. Gene specific DNA methylation is measured by the methylation assay (Kafri et al., 1992). In this method, total cellular DNA is digested with methyl-sensitive enzymes, such as HpaII or HhaI, and specific fragments of DNA that contain these sites are amplified by flanking oligonucleotide primers. If a specific site is methylated, the amplification will proceed normally. On the other hand, the presence of an unmethylated site will result in digestion of the fragment and the subsequent failure to visualize the amplification product. When properly calibrated, this assay is linear over a wide range of DNA concentrations and can be used to accurately measure the degree of DNA methylation at specific sites.

Identification of unique changes in gene expression by differential display

Total RNA is isolated from cells as described by Sambrook et al. The RNA is reverse transcribed with an oligo dT primer designed to bind to the 5' boundary of the poly A tail. The cDNA is amplified in a PCR reaction with the oligo dT primer and a second 10-mer arbitrary in sequence. 40 cycles of PCR are done in the presence of [35S]-dATP, in the following conditions: 94°C for 30 seconds, 42°C for 60 seconds and 72°C for 30 seconds. The amplified cDNAs were separated on a 6% sequencing gel, then exposed to X-ray film.

Bands of interest (bands that are differentially displayed) are cut out from the gel, and reamplified with the same primers as used to generate the original PCR product. To confirm differential regulation of individual candidate bands, Northern blot hybridizations is performed. Fragments of interest will be cloned using a TA cloning Kit, and sequenced.

Genes detected by this method are hybridized to Northern blots from the appropriate cells.

EXAMPLE OF STUDIES WITH AAV

A. Infection of mouse cells with AAV:

A1. Generation of mouse cells with stably integrated AAV:

The DA3 cell line was derived from the in-vivo D1-DMBA-3 mammary tumor syngeneic to BALB/C mice. The DA3 cell line produces tumors in BALB/C mice with the same growth kinetics and expresses the same tumor associated Ag on its surface as the parental tumor. The cells express specific markers for tumor cells, and cease to express specific Ag typical to normal breast cells (Sotomayor et al., 1991).

To assess the influence of AAV on mouse cells, DA cells were infected with the JDT277 AAV/neo hybrid virus (Tratschin, 1985). JDT277 contains the portion of the AAV2 genome, which encodes the Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882, resulting in carboxy terminus truncated Rep proteins. The truncation of the Rep proteins does not affect the ability of the AAV/neo virus to replicate in Adeonvirus coinfected human cells.

Single colonies were isolated and amplified by serial passages in the presence of G418. The resistant cells were designated DA35.

Generation of DA3 cells with stably integrated AAV:

DA3 cells were infected with the JDT277 AAV/neo hybrid virus according to Winocour et. al. (1992), with slight

modifications. Single colonies were isolated and amplified by serial passages in the presence of the antibiotic G418. The resistant cell lines were designated DA3J.

A2. Characterization of the AAV genome in the DA3J cells:

a. Southern analysis

Genomic DNA isolated from DA3J1 - DA3J7 clones was digested with different restriction enzymes (BglII or EcoRI), electrophoresed and hybridized to radiolabelled AAV DNA. The hybridization pattern is different in each clone, probably due to rearrangement of the AAV genome. Indeed it is known that integration of AAV DNA is frequently accompanied by alterations within the viral sequences (Walz and Schlehofer, 1992).

b. PCR analysis

To find whether the rep promoters and ORF's were present in the DA3J cell lines, PCR reactions were carried on 7 clones (DA3J1 - DA3J7), using different oligonucleotide primers complementary to the AAV and neo sequences. The results demonstrated that parts of the viral sequences in each clone were somehow interrupted. In all the examined cell lines the AAV rep ORF's were not intact, thus, impairing the expression of the AAV specific proteins.

In two clones, DA3J3 and DA3J4, two AAV molecules integrated into the host genome in a head-to- tail pattern. This finding is in agreement with earlier studies showing that the AAV DNA recombined into the Chinese hamster host DNA, at least in some cases, as a head-to-tail concatamer of more then one viral genome, via the terminal sequences of the viral molecule (Cheung et al., 1980; Walz and Schlehofer, 1992).

A3. Expression of AAV genes in the infected DA3J cells:

Cell extracts from the infected DA3 were prepared according to Winocour et al. (1992). Samples from the extracts were elctrophoresed on a 12% PAGE, and immunoblotted with an anti-Rep antibody. The results showed that, the two major Rep proteins, Rep78 and Rep68, are not expressed in any of the infected cells, however one of the small Rep proteins, Rep40, is expressed in two clones, DA3J11 and DA3J13. These results were expected from the PCR analysis that showed that in all the examined cell lines the rep ORF's were not intact.

B. Site specific integration

B1. Comparison of the integration site of AAV in the mouse cells with the AAV integration site in the Chinese hamster cells:

Genomic DNA from parental DA3 and DA3J clones (DA3J1 - DA3J7) was digested with BglII or EcoRI. Following electrophoresis the blots were hybridized once with the cellular sequence from the virus/cell junction, isolated from C9-3 (clone 6), and once with radiolabelled AAV DNA. In three of the cell lines (DA3J3, DA3J4 and DA3J6) the cellular probe and the AAV probe hybridized to common bands. Using PP2C probe applicant found in BglII digested DNA that both AAV and PP2C hybridized to the same bands. Thus, both in Chinese hamster and mouse AAV always integrated into the same site in the vicinity of PP2C. Note that in all cell lines including the parental DA3 cells. PP2C and the cellular clone 6 probes hybridized to the same band which is the preintegration site.

B2. Cloning the integration site of AAV in the DA3J cells:

Attempts to clone the flanking region of the integrated AAV from one of the DA3J clones, by inverse PCR were so far unsuccessful. Independent attempts to clone the flanking region of AAV from C9-3, by this method were also not successful. The difficulties arose probably due to the secondary structure at the ends of the AAV genome, which interfered with the polymerization by the Taq polymerase.

C. Effect of AAV on the cellular phenotype:

The following cytological properties were compared between DA3 infected and parental cells:

a. Plating efficiency

As shown in Table 1, the plating efficiency of the DA3J cells was reduced compared to the plating efficiency of the parental DA3 cells, by 11% (DA3J2) to 54% (DA3J3).

b. Sensitivity to UV irradiation

As shown in Table 2, the DA3J cells show increased sensitivity to UV irradiation compared to the parental DA3 cells. There is a decrease of 5% to 55% in the survival rate of the DA3J cells compared to the DA3 cells.

These results are in agreement with other studies, which demonstrated that AAV infected cells (Hela, C060) show reduced plating efficiency, and enhanced sensitivity to UV irradiation, compared to uninfected cells (Walz and Schlehofer, 1992; Winocour et al., 1992).

It is interesting to note that, DA3J3 shows the lowest plating efficiency, and the highest sensitivity to UV irradiation. This may be due to the fact that DA3J3 contains two integrated AAV molecules, while DA3J1 and DA3J2, contains only one.

c. FACS analysis

FACS analysis was performed on DA3, DA3J1, DA3J2, and DA3J3 as described by Vindelov et. al., (Vindelcv et al., 1983). In this procedure no significant changes were observed between the parental DA3 cells and the DA3J cells.

Throughout this application, various publications patents, are referenced by citation and patents by patent number. Full citations for the publications are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Table 1. Plating efficiency of the DA_3J clones compared with that of the parental DA_3 cell line

No. of cells plated	No. of outgrowing colonies (mean ± SD), % of plating efficiency			
	DA_3	DA_3J_1	DA_3J_2	DA_3J_3
250	88 (6) 35%	53 (8) 21%	78 (11) 31%	43 (5) 17%
500	181 (36) 36%	103 (10) 20%	131 (20) 26%	82 (9) 17%

Semiconfluent cells derived from DA_3, DA_3J_1, DA_3J_2, and DA_3J_3 cell cultures were seeded at 250 and 500 cells onto 9-cm plastic petri dishes. Cultures were grown for 7 days. Cells were than fixed and stained with Gimsa. The mean number of growing colonies from two experiments was determined. Each experiment was performed in triplicates.

Table 2. UV sensitivity of the DA_3J clones compared with that of the parental DA_3 cell line

J/m^2	No. of outgrowing colonies (mean ± SD), % of survival							
	DA_3		DA_3J_1		DA_3J_2		DA_3J_3	
0	91 (4)	100%	59 (20)	100%	79 (6)	100%	36 (7)	100%
2.5	86 (9)	95%	54 (9)	91%	85 (8)	100%	36 (4)	100%
5	62 (5)	68%	25 (30)	42%	39 (13)	49%	11 (11)	30%
10	22 (6)	24%	12 (2)	20%	19 (4)	24%	4 (2)	11%
20	0 (0)	0%	0 (0)	0%	2 (2)	2%	0 (0)	0%

Semiconfluently growing cells from DA_3, DA_3J_1, DA_3J_2, and DA_3J_3 cell cultures were seeded at 250 cells onto 9-cm plastic petri dishes. After 48 hours the cultures were irradiated with UV light, and then incubated for 7 days. The mean number of growing colonies was determined. The experiment was performed in triplicates.

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CLAIMS

What is claimed is:

1. A method of detecting cancer in a patient by detecting PP2C gene activity in a specimen containing cells isolated from the patient.

2. The method of claim 1 wherein the detecting of PP2C activity is by assaying the specimen for mRNA complementary to PP2C DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

3. The method of claim 1 wherein the detecting of PP2C activity is by assaying the specimen for a PP2C gene product including polymorphisms thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays and protein truncation test.

4. A kit for detecting PP2C activity, said kit comprising:

a molecular probe complementary to genetic sequences of a mRNA for PP2C including polymorphisms thereof and

detection means for detecting hybridization of said molecular probe and the mRNA thereby indicating the activity of the defective gene.

5. A kit for detecting a gene product associated with PP2C gene activity, said kit comprising:

an antibody which recognizes the PP2C gene product including polymorphisms thereof, and

detection means for detecting the binding of the antibody thereby indicating the presence of the gene product.

6. The kit as set forth in claim 5 further characterized by the detecting means utilizing agents which mimic natural proteins with which bind to the PP2C gene product.

7. A non-human transgenic mammal or cell line containing an expressible nucleic acid sequence for PP2C including polymorphisms thereof.

8. A non-human eucaryotic organism in which the equivalent nucleic acid sequence for PP2C is knocked-out.

9. A vector comprising an expression control sequence operatively linked to the nucleic acid sequence of PP2C.

10. A vector comprising an antisence sequence of PP2C.

11. An antibody which specifically binds to an epitope of a gene product of PP2C including polymorphisms thereof.

12. An antibody of claim 10 selected from the group consisting of monoclonal and polyclonal antibody.

13. An antibody of claim 11 conjugated to a detectable moiety.

14. A method of treating cancer including the steps of

a. determining the type of cancer and cells expressing the cancer,

b. preparing a vector which will specifically target the cancer cells including regulatory elements to control the expressibility of PP2C, and

c. administering the vector to the patient.

15. The method as set forth in claim 14 wherein the vector is a modified AAV.

16. The method as set forth in claim 14 wherein the vector contains the CHINT sequences.

17. A method of treating cancer including the steps of

a. determining the type of cancer and cells expressing the cancer,

b. preparing an antisense vector which will specifically target the cancer cells to control the expressibility of PP2C, and

c. administering the vector to the patient.

PR-314 (TAU)

MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION

ABSTRACT OF THE DISCLOSURE

A method of detecting cancer in a patient by detecting activity of the gene coding for human type protein phosphatase 2C (PP2C) and genetic polymorphisms thereof in a specimen containing cells isolated from the patient is disclosed. The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2C. The vector is then administered to the patient. Alternatively an antisense vector can be prepared.

FORM 7-4

PATENT

Attorney's Docket Number: PR-314 (TAU)

Applicant or Patentee: Sara Lavi

Serial or Patent No:

Filed or Issued: Herewith

For: MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)--SMALL BUSINESS CONCERN

I hereby declare that I am:

___ the owner of the small business concern identified below:

_X_ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern: Ramot-University Authority for Applied Research and Industrial Development, Ltd.

Address of Concern: 32 H. Jaavanon Street, P.O. Box 39296 Tel Aviv 61392, Israel

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement: (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when, either directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION

By Inventor(s): Sara Lavi
Described in:

_X_ the specification filed herewith.

___ application serial no. _____, filed _____.

___ patent no. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: _____

ADDRESS: _____

_____

___ Individual ___ Small Business ___ Nonprofit Organization

NAME: _____

ADDRESS: _____

_____

___ Individual ___ Small Business ___ Nonprofit Organization

I acknowledge the ducy to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Hanan Kvatinsky
Title in Organization: Assistant R&D Manager, Patents & Technology Transfer

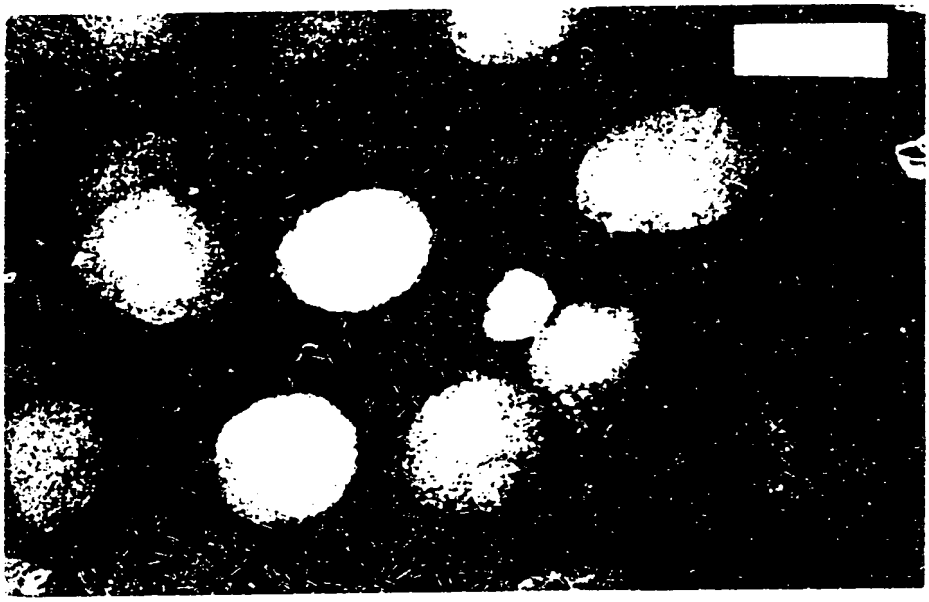
SIGNATURE: _____ Date: 9-1-95

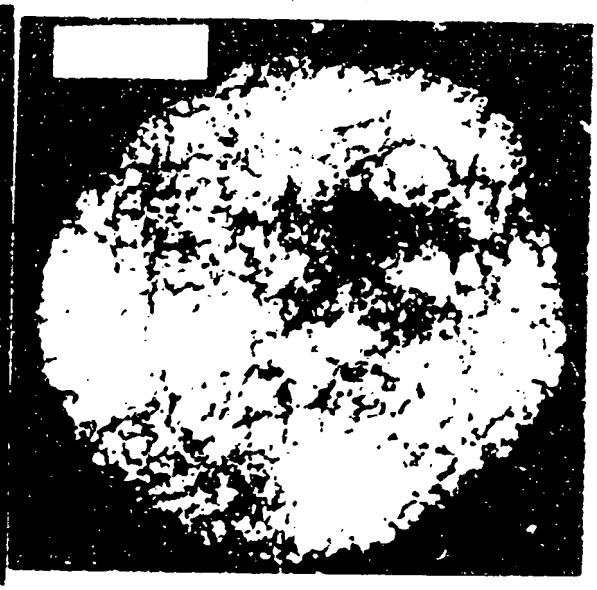
Name of Person Signing: Zvi Shoshan
Title in Organization: General Manager

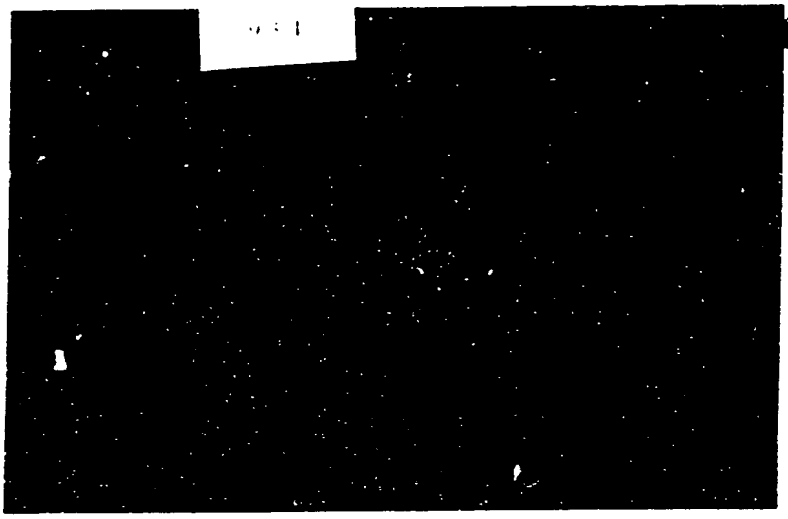
SIGNATURE: _____ Date: 9-1-95

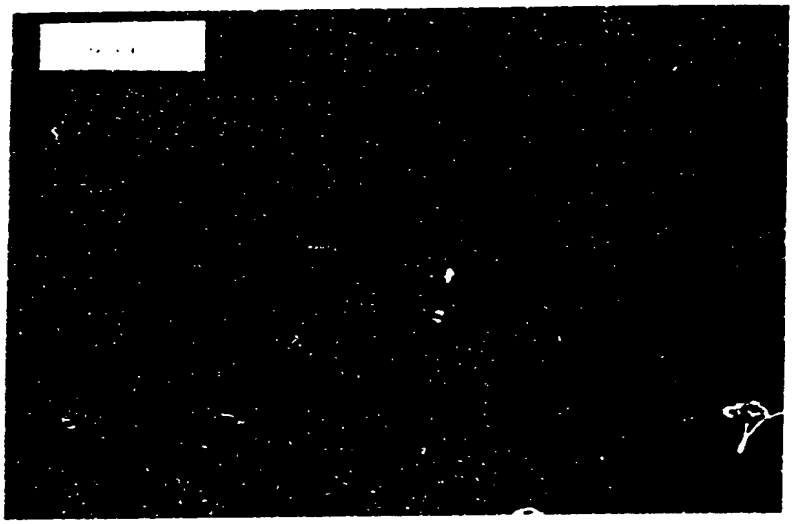
Address of Persons Signing: 22 Haim Levanon Street
Tel Aviv 61392, Israel

FORM 7-4

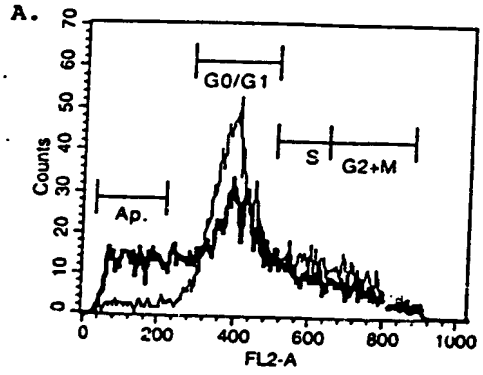








A.



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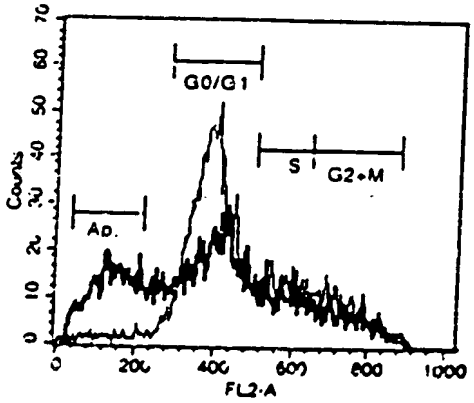
Total Events: 7000
Acquisition Date: 10-May-95
X Parameter: FL2-A (Linear)

Marker	Events	% Total	Mean	CV
All	7000	100.00	384.11	48.19
G0/G1	3355	47.93	394.68	14.49
S	943	13.47	589.28	7.41
G2+M	668	9.54	727.76	8.06
Ap.	1485	21.21	132.36	36.21

Key	Name	Parameter	Gate	
—	Shu..005	FL2-A	No Gate	Co60
—	Shu..003	FL2-A	No Gate	913

B.

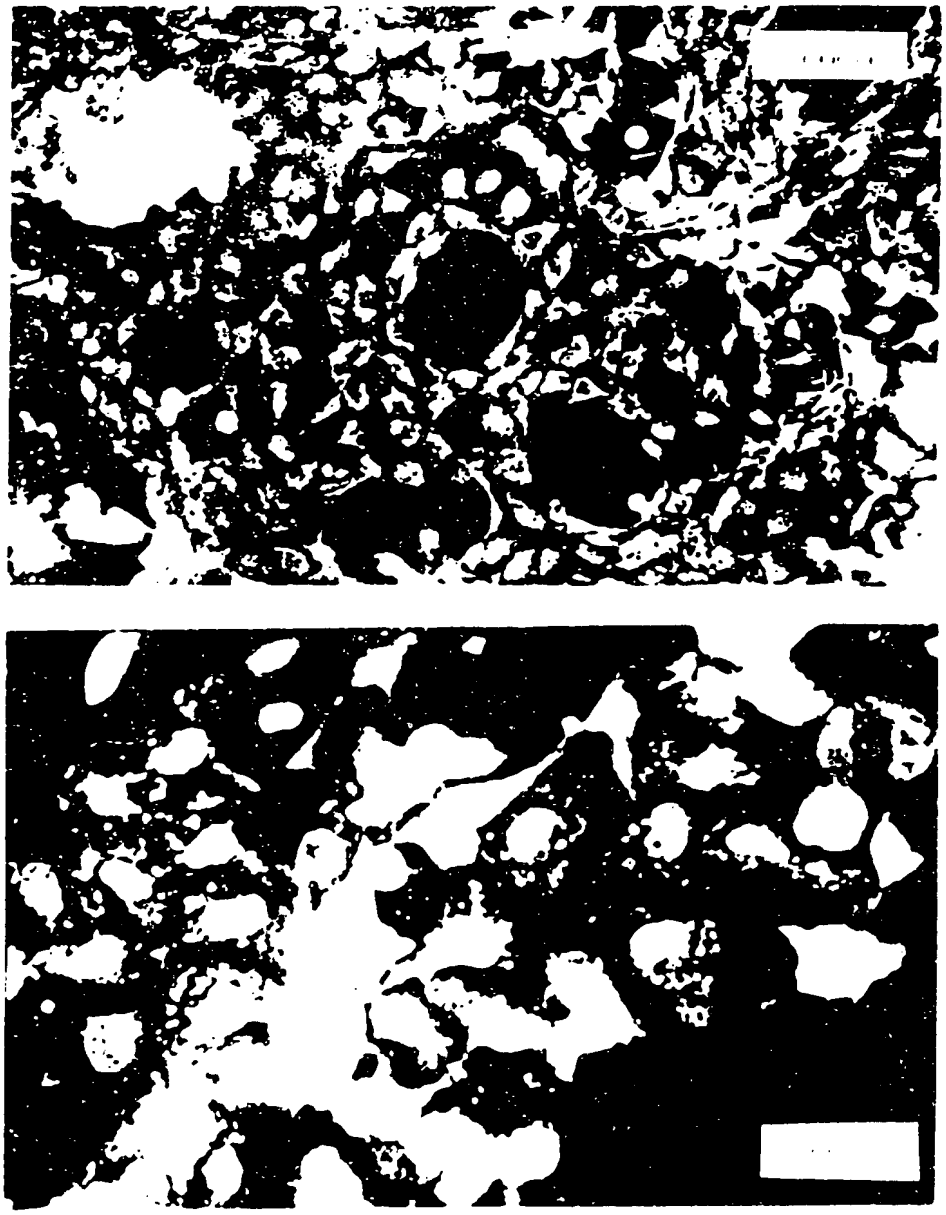


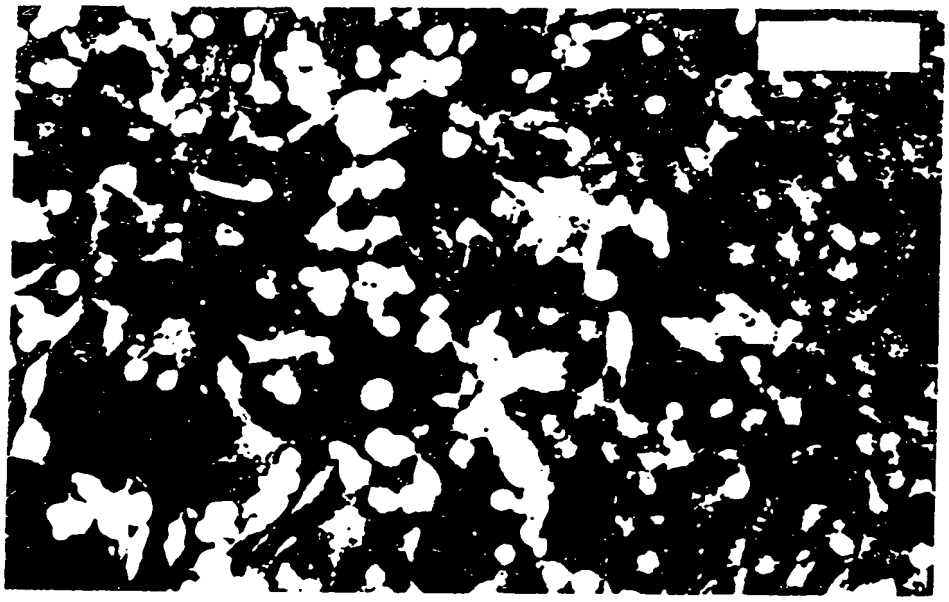
File: Shu..010
Total Events: 7000
Acquisition Date: 10-Ma
X Parameter: FL2-A (Lin

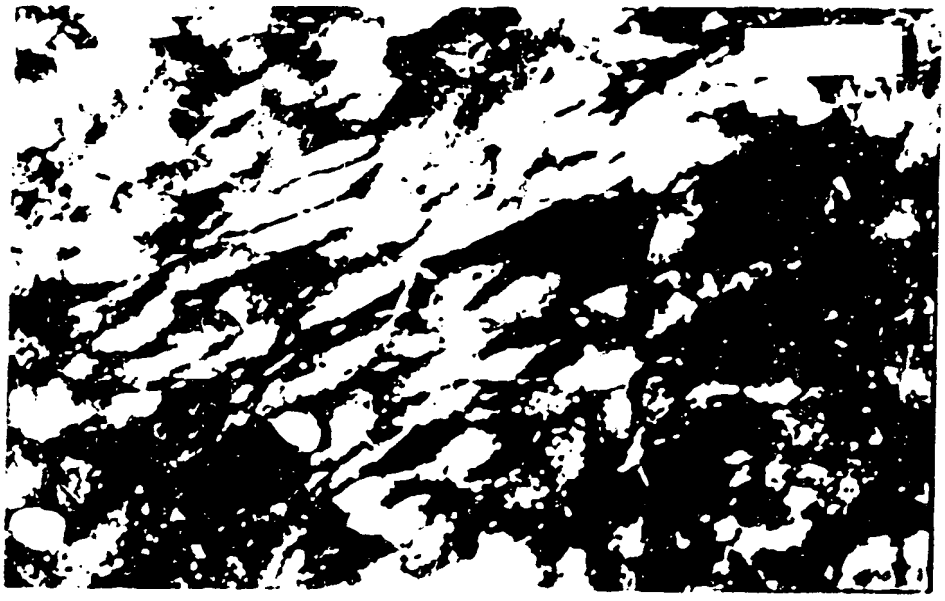
Marker	Events	% Total	Mean	CV
All	7000	100.00	362.46	51.50
G0/G1	3019	43.13	393.54	14.79
S	938	13.40	574.35	7.10
G2+M	798	11.40	737.43	8.24
Ap	1684	24.06	133.69	36.68

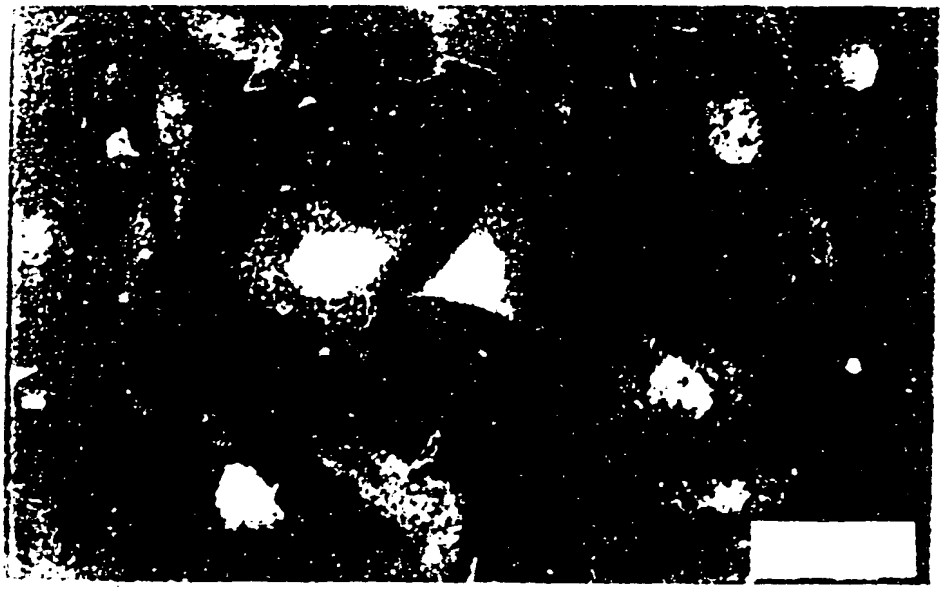
Key	Name	Parameter	Gate	
—	Shu 009	FL2-A	No Gate	Co60
—	Shu 010	FL2-A	No Gate	916

FIGURE 2









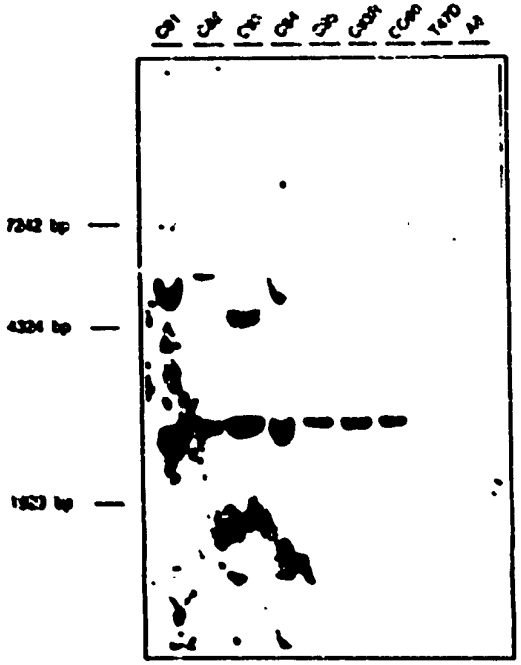


FIGURE 5

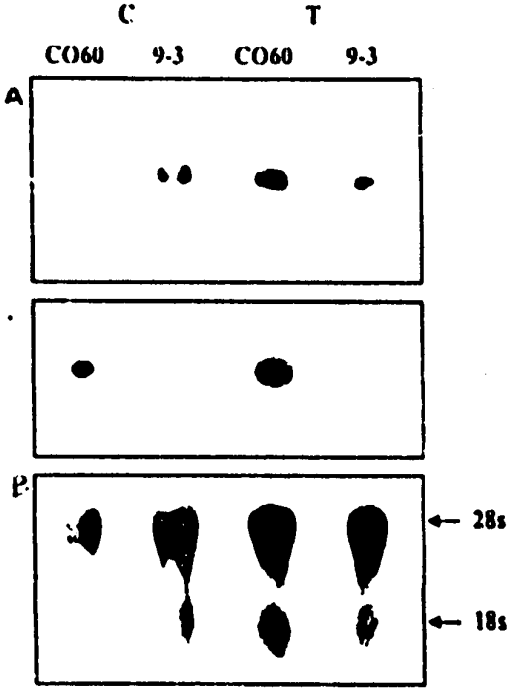
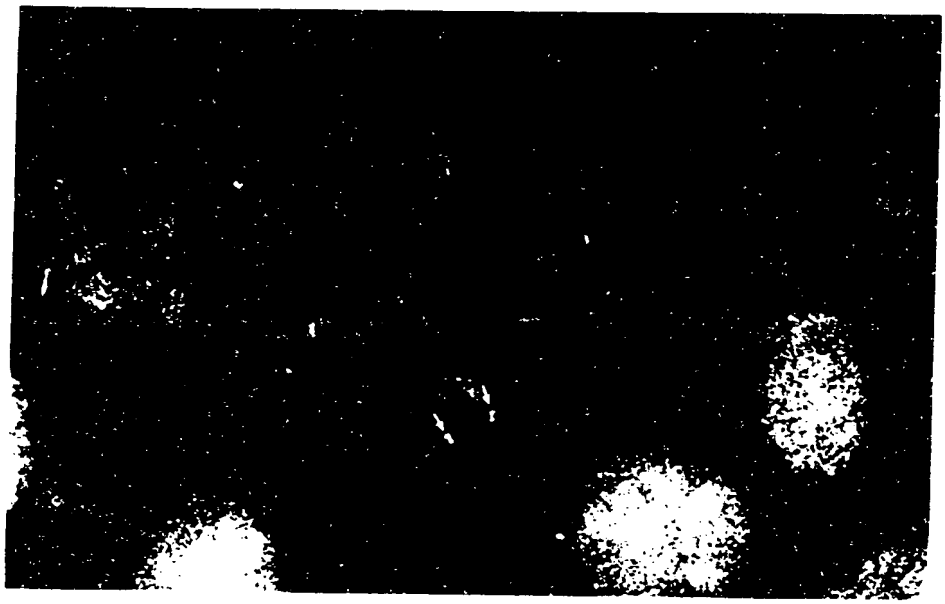


FIGURE 11

60/003114



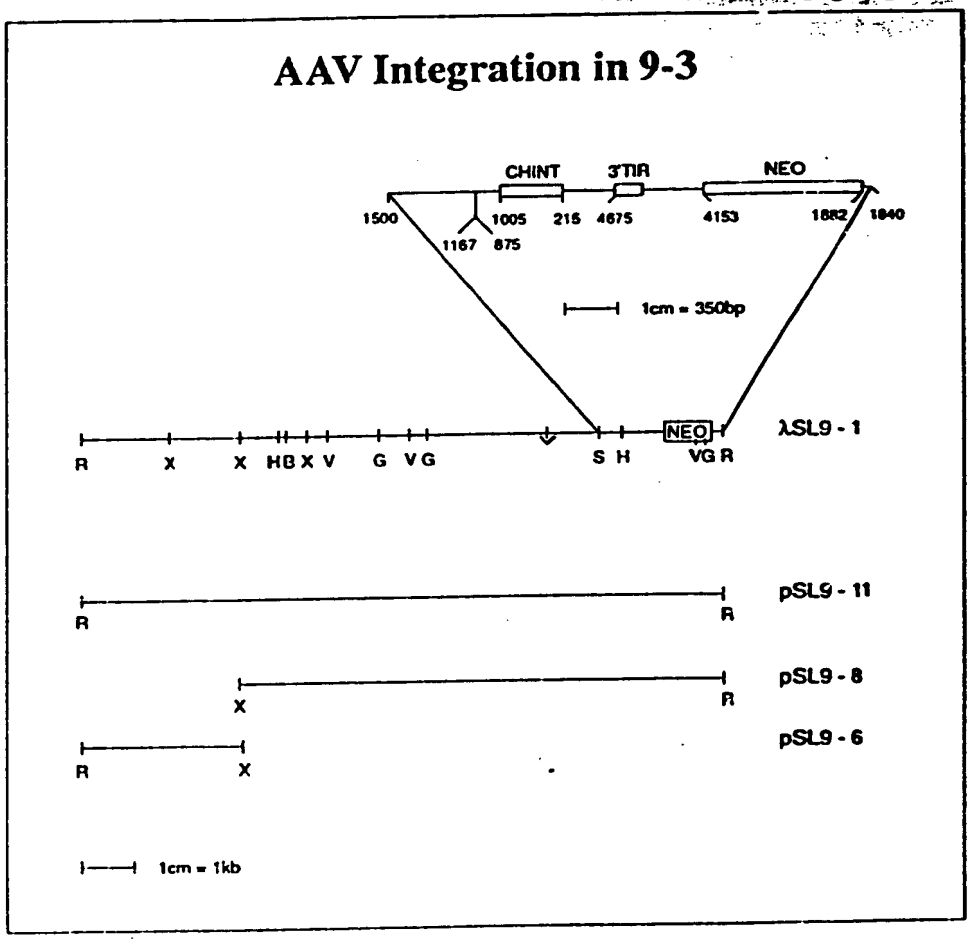

AAV Integration in 9-3
CHINT
3'TIR
NEO
1500
1005
215
4675
4153
1882
1840
1167
875
1cm = 350bp
NEO
λSL9 - 1
R
X
X
HB X V
G
V G
S H
VG R
pSL9 - 11
R
R
pSL9 - 8
X
R
pSL9 - 6
R
X
1cm = 1kb

FIGURE 7

```
ID   HSMMDBC     standard; DNA; PRI; 68505 BP.
AC   M89651; M77823; M77824; M77825;
DT   09-MAY-1992 (Rel. 31, Created)
DT   09-MAY-1992 (Rel. 31, Last updated, Version 1)
DE   Human DNA from cosmid DNA MMDB (f10080) and MMDC (f13544) from
DE   chromosome 19q13.3 (obtained by automated sequence analysis). . . .

SCORES      Init1: 61 Initn: 138 Opt: 82
             58.3% identity in 103 bp overlap

          20        30        40        50        60        70
int.1i TAGTGCCGGTCAAGGAACTGAACGTGCGATTCCGGGACAGGCTACCCACTCCGATCCCAG
          ||                |   |   |    |||||| ||||||||||  |  ||   |||
hsmmdb CCTCACCTCCGCCCTGTTTCGTCCAGGTCCTCCGGGTCAGGCTACCCCCGTCGCCGCCA-
       57710     57720     57730     57740     57750     57760

          80        90       100       110       120       130
int.1i GAGAAGTTGTCATGGTGAGGGCCACCCTAGGTCTCTGCCCCTGCTGTGTCCCCCCATCTTA
       |||  |  |    || ||| ||  || | |    ||  ||| ||    ||||||||
hsmmdb GAG-CGCGGGGGAGGGGAGAGCTTCCTTTGTCTCCTATGCCTCCT---CCCCCCATCCCG
         57770     57780     57790     57800          57810     57820

         140       150       160       170       180       190
int.1i CCCATCCAGTAGGATCTAGAGGCTGTCGCCCCCTTGTGGAATGCACAGAAGTCACAAGCG
       |  |||  |  ||      |  |  |   | ||  | | |  |||  |  |  |  |  |
hsmmdb GCTCTCCTGCGGGCAAGCGCCGAGGGGACACCGGGGAGTACCCCACCTGAACCTCTGGGG
          57830     57840     57850     57860     57870     57880
```

(GCG - Fasta)

FIGURE 8 A

60/003114

```
LOCUS HUMMMDB . 63505 bp ds-DNA PRI 09-APR-1992
Gaps: 16  Quality: 32.3  Ratio: 0.246  Score: 38  Width: 34  Limits: -

   16 GAGGGGGGGTGGGGGGAC....TCTAGACAGCTGAGGCGCGAAAGCAT 61
57842 GAGGGGACACCGGGGAGTACCCCACCTGAACCTCTGGGGCCC....ACAC 57887

   62 GAGTCCTCGGCTCTTCCTC..CTCCTT...............CTCCGGGA 94
57888 CAGGCCCCAG.TCTTCCTCTACTCACTGCAGTGGGCGAAGACCGGCAGGA 57936

   95 CCCGCTCTCT.GCCTCCCTC...............TCCAACGCCCGGATGA 129
57937 CCTGCCCCATGGCCCCCCTCGGGCCTGCATCGGGACCCCCGCCCACTCCG 57986

  130 TCTGAGCCG...............CGAGGGCGCCGA...CAGCCGGGGGC 161
57987 GCTGTGCCGCCCTGGGCCCGGGGTCGCGGGCGCTCATCTCCGCCGCGCCC 58036

  162 CCG..GACGCAGCCCGGCTCCTCCCCTCCTCCGCCC...CTTCCCCAGCC 206
58037 ACGACGCCGCTGCCATTCTCGCCGCCTCCTCCTCCCGGGCTGCTCCAG.. 58084

  207 TGACCTGGCCCGCCGCTGCAGCGGTGACCCCTCCCCCGGCTGCCGCCGTC 256
58085 .........CCGCCGCCG...............CCGCCGCCGCCGCCGCC 58110

  257 GCCGCCGCGGTGACCCCCTCCCCGGCTGCCGCCGCCGCC.......... 295
58111 GCCGCCCTGGTGCTCGGCTCGGCGGGGGCGGGCGGGGCCGAGGTGGGGAA 58160

  296 ......GCCTCGGCCGACCAGGGACCTGCC.......CGCCTGCGGCTGC 332
58161 GGCACTGCAGCGCGCGGCCGGGGGCGGGGCCAGAAGACGGGCGGGGCGGT 58210

  333 TCCGG......................ACCTAGAGGATCAAG 352
58211 ACCAGAGGGAGGGTGAGGGGCGGGGAAACGCCGAGGATCAGG 58252
```

FIGURE 8 B

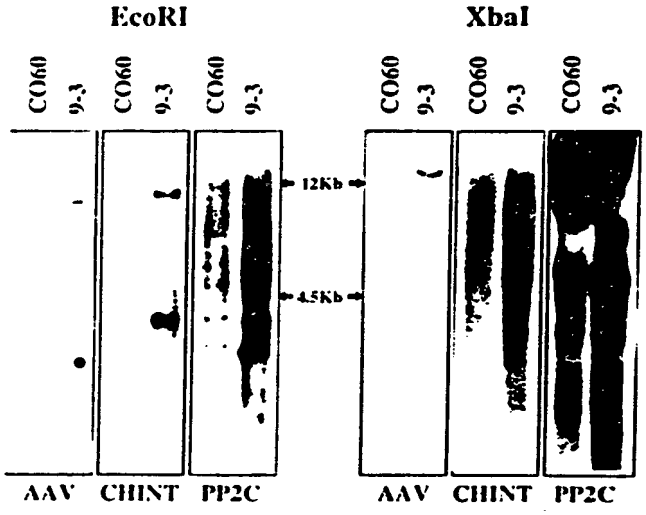


FIGURE 9 A

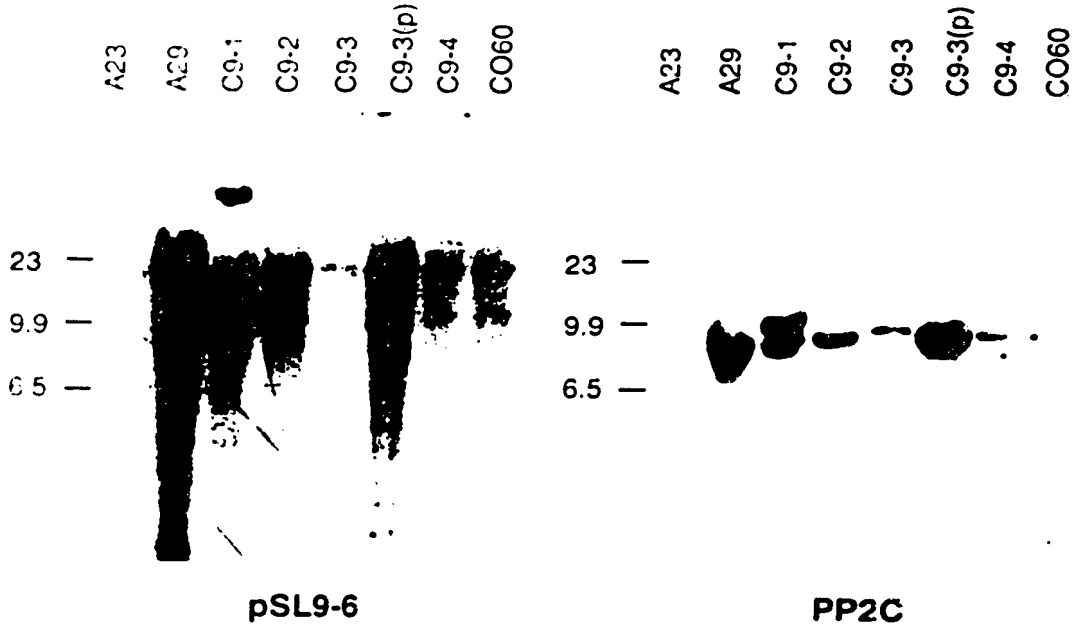


FIGURE 9 B

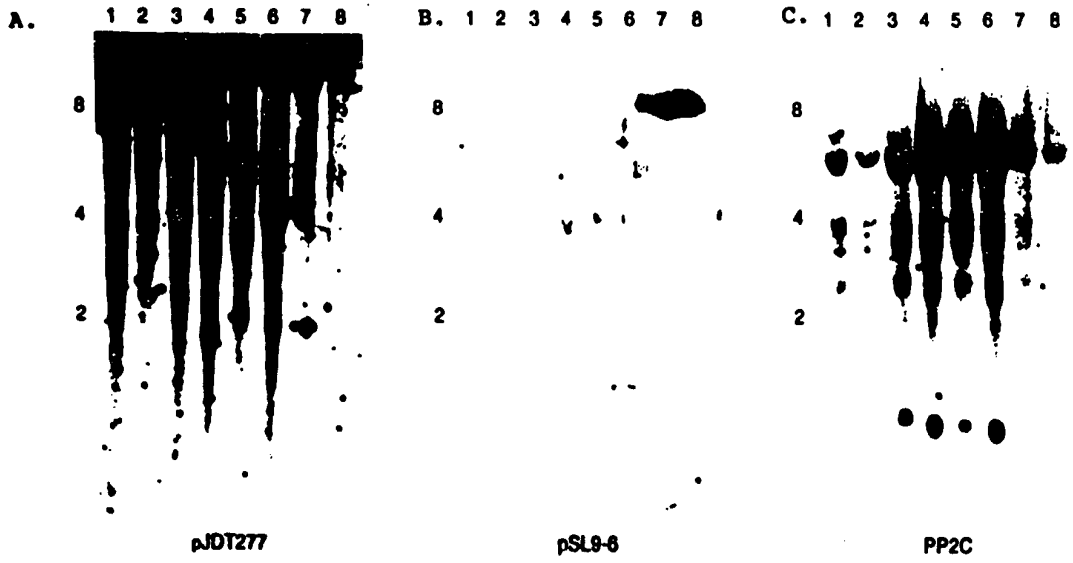


FIGURE 10